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Acyl homoserine lactone changes the abundance of proteins and the levels of organic acids associated with stationary phase in *Salmonella* Enteritidis

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ABSTRACT

Quorum sensing (QS) is cell-cell communication mechanism mediated by signaling molecules known as autoinducers (AIs) that lead to differential gene expression. *Salmonella* is unable to synthesize the AI-1 acyl homoserine lactone (AHL), but is able to recognize AHLs produced by other microorganisms through SdiA protein. Our study aimed to evaluate the influence of AI-1 on the abundance of proteins and the levels of organic acids of *Salmonella* Enteritidis. The presence of *N*-dodecyl-homoserine lactone (C12-HSL) did not interfere on the growth or the total amount of extracted proteins of *Salmonella*. However, the abundance of the proteins PheT, HtpG, PtsI, Adi, TalB, PmgI (or GpmI), Eno, and PykF enhanced while the abundance of the proteins RplB, RplE, RpsB, Tsf, OmpA, OmpC, OmpD, and GapA decreased when *Salmonella* Enteritidis was anaerobically cultivated in the presence of C12-HSL. Additionally, the bacterium produced less succinic, lactic, and acetic acids in the presence of C12-HSL. However, the concentration of extracellular formic acid reached 20.46 mM after 24 h and was not detected when the growth was in the absence of AI-1. Considering the cultivation period for protein extraction, their abundance, process and function, as well as the levels of organic acids, we observed in cells cultivated in presence of C12-HSL a correlation with what is described in the literature as entry into the stationary phase of growth, mainly related to nitrogen and amino acid starvation and acid stress. Further studies are needed in order to determine the specific role of the differentially abundant proteins and extracellular organic acids secreted by *Salmonella* in the presence of quorum sensing signaling molecules.

1. Introduction

Salmonella enterica serovar Enteritidis is the most common serotype responsible for salmonellosis in many countries and, this pathogen is primarily transmitted by food [1–4]. The high virulence of this pathogen is associated with the presence of many pathogenicity islands encoding virulence factors [5–7].

Quorum sensing (QS) is a mechanism of cell-cell communication mediated by signaling molecules known as autoinducers (AIs) that leads to differential gene expression in response to changes in the population density among microbial cells or microbial and host cells [8–11]. In *Salmonella*, this mechanism can be achieved through three types of autoinducers (AIs) called AI-1, AI-2, and AI-3. Complete sets of QS systems composed of signal synthase and signal receptors are present in many bacteria [12].

The QS system mediated by AI-1 is present in Gram-negative

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bacteria, but in *Salmonella* it is incomplete. This pathogen is unable of synthesizing the AI-1 called acyl homoserine lactone (AHL) since neither *luxI* gene nor other homologues that codify for the AI-1 synthase are present in the bacterial genome. However, *Salmonella* is able to recognize AHLs synthesized by other microorganisms through SdiA protein, a transcriptional regulator homologous to LuxR which is the signal receptor [13–15]. The AHLs are internalized and bind to the ligand-binding domain (LBD) of SdiA which dimerizes and binds to DNA by using its DNA-binding domain (DBD) regulating expression of target genes [16]. The AI-2 is found in *Salmonella* where it is synthesized by LuxS and internalized by using products of the *lsr* operon [17–19]. In the QS mediated by AI-3 in *Salmonella*, the signal molecules are the hormones epinephrine and norepinephrine, synthesized by animal cells, which are sensed through proteins coded by the *qseBC* operon and *qseE* gene [12,20].

Autoinducers influence gene expression and protein abundance in *Salmonella,* consequently generating different phenotypes, including motility, biofilm formation as well as adhesion, invasion and survival in eukaryotic cells [12,15,21–27]. Campos-Galvão et al. [26] showed enhanced biofilm formation by *Salmonella* Enteritidis PT4 578 growing in the presence of 50 nM of AHLs with six, eight, ten and twelve carbons. However, the effect of *N*-dodecanoyl-DL-homoserine lactone (C12-HSL) on this phenotype was statistically higher than the other AHLs evaluated. These data were reinforced by the recently published study on molecular docking in which AHLs with twelve carbons presented greater affinity to SdiA of *Salmonella* Enteritidis PT4 578 than AHLs with ten, eight, six and four carbons side chains [28]. Liu et al. [25] reported that the presence of AI-1 increased the adhesion of *S. enterica* serovar Typhi to HeLa cells and biofilm formation in polystyrene. Similar results were observed for *Salmonella* Enteritidis in presence of AI-1, where biofilm formation in polystyrene was increased when cells were cultivated in anaerobic conditions [26,27]. Moreover, the *rck* operon of *Salmonella*, which is related to virulence, was more expressed in presence of the AI-1 as previously observed by other groups [14,29]. So far, only two studies evaluated the influence of QS in the abundance of proteins in *Salmonella*. In both cases, the effect of AI-2 on two strains of *S. enterica* serovar Typhimurium was tested, but no studies evaluated the influence of AI-1 on this bacterium [30,31].

Proteomics allows the determination of the global picture of proteins expressed by the genome and gives new insights into the behavior of bacteria during the QS phenomena [32]. Conventional two-dimensional gel electrophoresis (2-DE) in combination with advanced mass spectrometric techniques has facilitated the characterization of thousands of proteins using a single polyacrylamide gel. The 2-DE procedure allows easy visualization of protein isoforms and posttranslational modifications (PTMs) based on protein separation using two physical parameters such as isoelectric point and molecular weight, rendering this technology extremely informative [33].

Thus, considering the scarcity of information about the influence of AI-1 in *Salmonella*, our study aimed to evaluate the effect of this signaling molecule in the abundance of proteins and the levels of organic acids of *Salmonella* Enteritidis. The comparative analysis helps to understand the QS mechanism dependent upon AHL on the physiology of this pathogen.

2. Materials and methods

2.1. Bacterial strain

Salmonella enterica sorovar Enteritidis PT4 578, isolated from chicken meat, was provided by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil) and has been previously described [26,27,34]. Cultures were stored at −20 °C in Luria-Bertani (LB) broth [35] supplemented with 20% (v/v) of sterile glycerol.

2.2. Preparation of inoculum

Tryptone soy broth (TSB; Merck, Germany) was prepared under O_2-free conditions with a CO_2 filling and was dispensed into anaerobic bottles that were sealed with butyl rubber stoppers and then, autoclaved (anaerobic TSB) [36]. Before each experiment, cells were cultivated in anaerobic bottles containing 20 mL of anaerobic TSB for 24 h at 37 °C in a static-model anaerobic chamber (Coy Laboratory, USA) containing a mixture of H_2 (3–5%) and CO_2 (95–97%). Then, 1 mL of culture was transferred into 10 mL of anaerobic TSB and incubated at 37 °C in anaerobic chamber. After incubation for 4 h, exponentially growing cells were harvested by centrifugation at 5000*g* at 4 °C for 10 min (Sorvall, USA), washed with 0.85% saline, and the pellet resuspended in 0.85% saline. The inoculum was standardized to 0.1 of optical density at 600 nm (OD_{600nm}), approximately 10^7 colonies forming units per milliliter (CFU/mL), using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.3. Preparation of HSL solution

N-dodecanoyl-DL-homoserine lactone (C12-HSL; PubChem CID: 11565426; Fluka, Switzerland) was suspended in acetonitrile (PubChem CID: 6342; Merck, Germany) at a concentration of 10 mM and further diluted to a working solution of 10 μM in acetonitrile. Control experiment was performed using acetonitrile. The final concentration of acetonitrile in the media was always less than 1% (v/v) to avoid interference in the growth and response of *Salmonella* to C12-HSL [14].

2.4. Effect of HSL on the growth of Salmonella

To evaluate the effect of C12-HSL on the growth of *Salmonella*, bottles containing 20 mL of anaerobic TSB supplemented with 50 nM of C12-HSL were inoculated with 2 mL of the standardized inoculum. Bottles were incubated at 37 °C for up to 12 h in anaerobic chamber. In established time points, the OD_{600nm} was determined using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.5. Extraction and quantification of proteins of Salmonella

A standardized inoculum was added into anaerobic bottles containing 30 mL of anaerobic TSB supplemented with 50 nM of C12-HSL or the equivalent volume of acetonitrile as control and then, incubated at 37 °C in anaerobic chamber. After 7 h of incubation, the OD_{600nm} and CFU/mL were determined. Concomitantly, an aliquot of the media was centrifuged at 5000*g* at 4 °C for 15 min (Sorvall, USA). The cells in the pellet were resuspended in 1 mL of sterilized distilled water, transferred to 1.5 mL microtubes and once again centrifuged at 9500*g* at 4 °C for 30 min (Brikmann Instruments, Germany). The pellet was resuspended in 50 mM ammonium bicarbonate, 1 mM phenylmethylsulfonyl fluoride (PMSF) added of 1 mL of 2:1 trifluoroethanol:chloroform (TFE:$CHCl_3$), followed by vigorous agitation. Next, the mixture was kept at 0 °C for 1 h in ultrasound bath (100 W MSE 20 KHz), with mixing every 10 min. The material was centrifuged at 6500*g* at 4 °C for 4 min (Brikmann Instruments, Germany) to obtain three phases. The upper phase (composed by proteins soluble in TFE) and the central phase (composed by proteins insoluble in TFE) were collected. The mixture of both phases were dried in SpeedVac (Genevac, England) and resolubilized in 500 μL of a mixture of 5 M urea, 2 M tiourea, 4% (w/v) 3-[(3-cholamidopropyl)